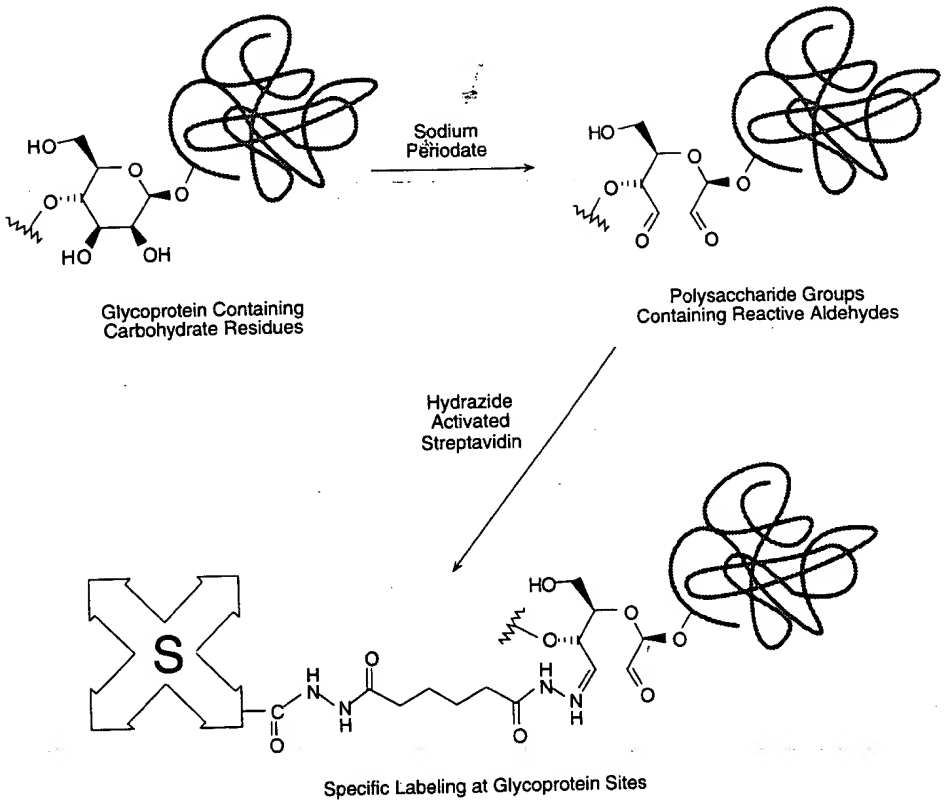

Figure 369 Glycoproteins may be oxidized with sodium periodate to generate aldehyde residues. These may be specifically labeled using a hydrazide–streptavidin derivative through hydrazone bond formation. Subsequent detection may be done using biotinylated enzymes.

Protocol

1. In a test tube, dissolve 160 mg of adipic acid dihydrazide (Aldrich) in 5 ml of 0.1 *M* sodium phosphate, pH 6. Some heating of the tube under a hot-water tap may be required to help solubilize the compound. Cool to room temperature.
2. Dissolve 50 mg of avidin or streptavidin (Pierce) in the adipic acid dihydrazide solution.
3. Add 160 mg of the water-soluble carbodiimide EDC (Pierce) (Chapter 3, Section 1.1) to the solution, and mix to dissolve.
4. React for 4 h at room temperature.
5. Dialyze against PBS, pH 7.2 to remove excess reagent and reaction by-products.

Hydrazide-activated avidin or streptavidin may be stored as a freeze-dried preparation without loss of activity.

6. Biotinylation Techniques

In addition to preparing the avidin or streptavidin conjugates necessary to develop avidin–biotin-based systems, the process of modifying targeting molecules with a

biotin tag is just as critical and forms the other key component of the interacting complex. Since biotin is a relatively small molecule (MW 244.31), coupling it to macromolecules usually can be done without disturbing the activity or binding capability of either the targeting molecule or the biotin handle. Proteins, carbohydrates, lipid molecules, and nucleic acids can be modified to contain one or more biotins able to interact strongly with avidin or streptavidin. The technique of biotinylation is made easy through the commercial availability of a range of different biotin derivatives having a number of important reactivity and property characteristics useful in avidin–biotin chemistry.

Chapter 8, section 3, describes the major biotinylation compounds and their properties. Also provided in that section are suggested protocols for reacting each of these reagents with specific functional groups on macromolecules.

7. Determination of the Level of Biotinylation

It is often important to determine the extent of biotin modification after a biotinylation reaction is complete. Measuring biotin incorporation into macromolecules can aid in optimizing a particular avidin–biotin assay system, and it also can be used to ensure reproducibility in the biotinylation process. The most common method of measuring the degree of biotinylation makes use of the 4′-hydroxyazobenzene-2-carboxylic acid (HABA) dye assay (Green, 1965). In the absence of biotin, the dye is capable of specifically forming noncovalent complexes with avidin at its biotin-binding sites. On binding to avidin in aqueous solution, HABA exhibits a characteristic absorption band at 500 nm ($\varepsilon = 35{,}500\ M^{-1}cm^{-1}$, expressed as per mole of HABA bound). The addition of biotin to this complex results in displacement of HABA from the binding site, since the affinity constant of the avidin–biotin interaction ($1.3 \times 10^{15}\ M^{-1}$) is much greater than that for avidin–HABA ($6 \times 10^{6}\ M^{-1}$). As HABA is displaced, the absorbance of the complex decreases proportionally. Thus, the amount of biotin present in the solution can be determined by plotting the avidin–HABA absorbance at 500 nm versus the absorbance modulation with increasing concentrations of added biotin. Comparing an unknown biotin-containing sample to this standard response curve can result in the determination of the biotin concentration in the sample.

Since a biotinylated molecule is able to interact with avidin at its biotin binding sites just as strongly as biotin in solution, the degree of biotinylation may be determined using the HABA method as well. Comparison of the response of a biotinylated protein, for example, with a standard curve of various biotin concentrations allows calculation of the molar ratio of biotin incorporation.

Two variations of HABA dye assay for biotinylated proteins are possible. In one approach, the biotinylated protein is digested using the enzyme pronase prior to doing the assay. The digestion process breaks the protein into small fragments, some of which possess biotin modifications. The digestion is done to eliminate any sterically hindered biotinylation sites from not being able to interact with avidin. The second approach merely uses the intact biotinylated protein in the assay, assuming that the HABA assay results then will provide a truer picture of the level of *accessible* biotin sites on the molecule. Pronase addition is obviously not necessary for assessing biotinylated molecules that are not proteins.

The following protocol describes both of these HABA-based tests for determining the level of biotinylation.

Protocol

1. Dissolve avidin in 0.05 *M* sodium phosphate, 0.15 *M* NaCl, pH 6, at a concentration of 0.5 mg/ml. A total of 3 ml of the avidin solution is required to create a standard curve using known concentrations of biotin and an additional 3 ml is needed for each sample determination.
2. Dissolve the HABA dye (Sigma) in 10 m*M* NaOH at a concentration of 2.42 mg/ml (10 m*M*). Prepare about 100 µl of the HABA solution for each 3-ml portion of avidin solution required.
3. Dissolve the biotinylated protein to be measured in 0.05 *M* sodium phosphate, 0.15 *M* NaCl, pH 6, at a concentration of 10–20 mg/ml. The amount required is about 100 µl of sample per determination.
4. Dissolve D-biotin (Pierce) in 0.05 *M* sodium phosphate, 0.15 *M* NaCl, pH 6, at a concentration of 0.5 m*M*.
5. For the proteolytic digestion procedure, dissolve pronase in water at a concentration of 1% (w/v).
6. If pronase digestion of the biotinylated protein is to be done, heat 100 µl of the sample at 56°C for 10 min, then add 10 µl of the pronase solution. Allow the sample to enzymatically digest at room temperature overnight. If no pronase digestion is desired, simply use the biotinylated protein solution prepared in step 3 without further treatment.
7. To construct a standard curve of various biotin concentrations, first zero a spectrophotometer at an absorbance setting of 500 nm with both sample and reference cuvettes filled with 0.05 *M* sodium phosphate, 0.15 *M* NaCl, pH 6. Remove the buffer solution from the sample cuvette and add 3 ml of the avidin solution plus 75 µl of the HABA dye solution. Mix well and measure the absorbance of the solution at 500 nm. Next add 2-µl aliquots of the biotin solution to this avidin–HABA solution, mix well after each addition, and measure and record the resultant absorbance at 500 nm. With each addition of biotin, the absorbance of the avidin–HABA complex at 500 nm decreases. The absorbance readings are plotted against the amount of biotin added to construct the standard curve.
8. To measure the response of the biotinylated protein sample, add 3 ml of the avidin solution plus 75 µl of the HABA dye to a cuvette. Mix well and measure the absorbance of the solution at 500 nm. Next add a small amount of sample to this solution and mix. Record the absorbance at 500 nm. If the change in absorbance due to sample addition was not sufficient to obtain a significant difference from the initial avidin–HABA solution, add another portion of sample and measure again. Determine the amount of biotin present in the protein sample by using the standard curve. The number of moles of biotin divided by the moles of protein present gives the number of biotin modifications on each protein molecule.